

## HER-2 BINDING ANTAGONISTS

### Technical Field of the Invention

The present invention provides a HER-2 binding antagonist. Specifically, intron  
5 retention has generated a novel HER-2 antagonist polypeptide that binds to the HER-2 receptor.

This work was supported by a grant from the Department of Defense (DOD) Breast  
Cancer Research Program. The United States Government has certain rights in this invention.

### Cross-reference to Related Applications

This application is a continuation-in-part of United States Patent Application Serial No.  
10 09/234,208, entitled HER-2 BINDING ANTAGONISTS, filed 20 January 1999.

### Background of the Invention

The HER-2/neu (erbB-2) oncogene encodes a receptor-like tyrosine kinase (RTK) that  
has been extensively investigated because of its role in several human carcinomas (Hynes and  
Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-  
15 2123, 1994) and in mammalian development (Lee et al., *Nature* 378:394-398, 1995). The  
sequence of the HER-2 protein was determined from a cDNA that was cloned by homology to  
the epidermal growth factor receptor (EGFR) mRNA from placenta (Coussens et al., *Science*  
230:1132-1139, 1985) and from a gastric carcinoma cell line (Yamamoto et al., *Nature* 319:230-  
234, 1986). The HER-2 mRNA was shown to be about 4.5 kb (Coussens et al., *Science*  
20 230:1132-1139, 1985; and Yamamoto et al., *Nature* 319:230-234, 1986) and encodes a  
transmembrane glycoprotein of 185 kDa in normal and malignant human tissues (p185HER-2)  
(Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene*  
9:2109-2123, 1994). The function of the HER-2 gene has been examined mainly by expressing  
the cDNA corresponding to the 4.5 kb transcript in transfected cells and from the structure and  
25 biochemical properties of the 185 kDa protein product. P185HER-2 consists of a large  
extracellular domain, a transmembrane segment, and an intracellular domain with tyrosine kinase  
activity (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al.,  
*Oncogene* 9:2109-2123, 1994). Overexpression of p185HER-2 causes phenotypic  
transformation of cultured cells (DiFiore et al., *Science* 237:178-182, 1987; and Hudziak et al.,  
30 *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987) and has been associated with aggressive clinical  
progression of breast and ovarian cancer (Slamon et al., *Science* 235:177-182, 1987; and Slamon  
et al., *Science* 244:707-712, 1989). p185HER-2 is highly homologous to the EGFR. However, a  
ligand that directly binds with high affinity to p185HER-2 has not yet been identified.  
Moreover, the signaling activity of HER-2 may be mediated through heterodimerization with  
35 other ligand-binding members of the EGFR family (Carraway and Cantley, *Cell* 78:5-8, 1994;  
Earp et al., *Breast Cancer Res. Treat.* 35:115-132, 1995; and Qian et al., *Oncogene* 10:211-219,

1995).

Divergent proteins, containing regions of the extracellular domains of HER family RTKs, are generated through proteolytic processing of full length receptors (Lin and Clinton, *Oncogene* 6:639-643, 1991; Zabrecky et al., *J. Biol. Chem.* 266:1716-1720, 1991; Pupa et al., *Oncogene* 8:2917-2923, 1993; Vecchi et al., *J. Biol. Chem.* 271:18989-18995, 1996; and Vecchi and Carpenter, *J. Cell Biol.* 139:995-1003, 1997) and through alternative RNA processing (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990; Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993; and Lee and Maihle, *Oncogene* 16:3243-3252, 1998). The extracellular domain of p185HER-2 is proteolytically shed from breast carcinoma cells in culture (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990; Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993; and Lee and Maihle, *Oncogene* 16:3243-3252, 1998), and is found in the serum of some cancer patients (Leitzel et al., *J. Clin. Oncol.* 10:1436-1443, 1992) where it may be a serum marker of metastatic breast cancer (Leitzel et al., *J. Clin. Oncol.* 10:1436-1443, 1992) and may allow escape of HER-2-rich tumors from immunological control (Baselga et al., *J. Clin. Oncol.* 14:737-744, 1996; and Brodowicz et al., *Int. J. Cancer* 73:875-879, 1997).

A truncated extracellular domain of HER-2 is also the product of a 2.3 kb alternative transcript generated by use of a polyadenylation signal within an intron (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). The alternative transcript was first identified in the gastric carcinoma cell line, MKN7 (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993) and the truncated receptor was located within the perinuclear cytoplasm rather than secreted from these tumor cells (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). However, no particular therapeutic, diagnostic or research utility has been ascribed to this truncated extracellular domain polypeptide. A truncated extracellular domain of the EGFR, generated by alternative splicing (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990) is secreted, exhibits ligand-binding, and dimerization properties (Basu et al., *Mol. Cell. Biol.* 9:671-677, 1989), and may have a dominant negative effect on receptor function (Basu et al., *Mol. Cell. Biol.* 9:671-677, 1989; and Flickinger et al., *Mol. Cell. Biol.* 12:883-893, 1992).

Therefore, there is a need in the art to find molecules that bind to cellular HER-2 and particularly molecules that bind to different sites than humanized antibodies to HER-2 (e.g., Herceptin®). Such molecules would be useful therapeutic agents for various cancers that overexpress HER-2.

### Summary of the Invention

The present invention provides an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide

binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2).

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The present invention further provides an isolated DNA sequence that codes, on  
5 expression, for a polypeptide having from about 50 to 79 amino acids taken from the sequence of  
SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an  
affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in  
length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different  
10 from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used  
for the treatment of cancer and that binds to the ECD or HER-2). The present invention further  
provides a transfected cell comprising an expression vector having a DNA sequence that codes  
on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence  
of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at  
an affinity of at least  $10^8$ .

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15 The present invention further provides an isolated and glycosylated polypeptide having  
from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C  
terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are  
present. Preferably, the isolated polypeptide is from about 350 to 419 amino acids in length and  
four N-linked glycosylation sites are present. Preferably, the isolated polypeptide binds to a site  
20 on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed  
humanized monoclonal antibody that is used for the treatment of cancer and that binds to the  
ECD or HER-2).

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The present invention further provides an isolated DNA sequence that codes on  
25 expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence  
of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three  
N-linked glycosylation sites are present. Preferably, the isolated polypeptide is from about 350  
to 419 amino acids in length and four N-linked glycosylation are present. The present invention  
further provides a transfected cell comprising an expression vector having a DNA sequence that  
codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the  
30 sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at  
least three N-linked glycosylation sites are present.

35 The present invention provides a method for treating a solid tumor characterized by  
overexpression of HER-2, comprising administering an agent that binds to the extracellular  
domain (ECD) of HER-2, wherein the agent is selected from the group consisting of (a) an  
isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID  
NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of

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at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone. Preferably, the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer and colon cancer. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The present invention further provides a pharmaceutical composition for treating tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The present invention further provides a method for targeting a therapeutic agent to solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD of HER-2).

The present invention further provides a method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic

fluid, chorionic villi tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; (b) determining whether a particular ECDIIIa variant sequence is present in the bodily fluid sample with a sequence identity assay; and (c) correlating the presence of the ECDIIIa variant sequence to cancer treatment and diagnosis using an historical database. Preferably, the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; (b) determining whether an amount of an p68HER-2 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample, and determining a ratio between the amount of p185HER-2 ECD and a particular p68HER-2 ECDIIIa variant.

The present invention further provides for antibodies specific for ECDIIIa variants of the sequence in SEQ ID NO:1 or SEQ ID NO:2, below.

## Brief Description of the Drawings

Figure 1 shows a nucleotide and amino acid sequence of the insert in the extracellular domain of HER-2. The HER-2 ECD coding sequence from exon 1-9 (primers A and B) was amplified by PCR from a cDNA library from SKOV-3 cells. A product of ~1420 bp was found to be HER-2-specific by Southern blot analysis. This product was subcloned and the nucleotide sequence was determined. In panel A, the nucleotide sequence is shown for the 274 bp insert (outside the box) and for the immediately adjacent 5' and 3' sequences enclosed in the box. The insert is located between nucleotide residues 1171 and 1172 and following amino acid residue 340 in p185HER-2 using the numbering of Coussens et al. (*Science* 230:1132-1139, 1985). The consensus 5' and 3' splice sites at the arrows are shown in larger print. The inserted sequence is in-frame with 5' HER-2 exon sequence and is deduced to encode a 79 amino acid extension following Arg 340 (R<sup>340</sup>). The novel 79 novel amino acid sequence encoded by the insert is proline-rich (19%) and has a consensus asparagine linked glycosylation site, which is underlined.

A stop codon was found at nucleotides 236-238 within the inserted sequence. In panel B, the predicted product of the alternative transcript is a truncated secreted protein which contains subdomains I and II identical to p185 and is missing the transmembrane domain and cytoplasmic domain. If fully glycosylated, the expected size is 65-70 kDa. This polypeptide product is referred to as p68HER-2. Thus, the product will be a truncated secreted protein which is missing the transmembrane domain and cytoplasmic domain found in p185HER-2.

Figure 2 shows the detection of alternative HER-2 transcripts containing the ECDIIIa sequence by Northern blot analysis. PolyA<sup>+</sup> mRNA (2.5 µg) from different human fetal tissues (Clontech) or isolated from HEK-293 cells was resolved in a formalin agarose gel and transferred to a BrightStar® membrane (Ambion) in 10xSSC. The membrane was hybridized with a <sup>32</sup>P-labeled antisense RNA probe complimentary to the ECDIII sequence, stripped and reprobed with a <sup>32</sup>P-labeled cDNA probe specific for the 5' HER-2 exon sequence. The membranes were washed under high stringency conditions and analyzed by phosphorimaging (Molecular Dynamics).

Figure 3 shows a sequence-specific reactivity of anti-ECDIIIa with a protein of ~ 68 kDa in a human embryonic kidney cell line (HEK293). Cell extract protein (20 µg) and 20 µl of media conditioned by HEK-293 cells were Western blotted and probed with anti-ECDIIIa diluted 1:10,000 (lanes 1 and 2) or with anti-ECDIIa diluted 1:10,000 containing 50 µg/ml purified His-tagged ECDIIIa peptide (lanes 3, 4).

Figure 4 shows the expression of p185HER-2, relative to p68ECDIIIa expression, is markedly elevated in carcinoma cell lines in which the HER-2 gene is amplified. Cell extracts (15 µg of protein) from human embryonic kidney cell line (HEK293), nontumorigenic ovarian surface epithelial cell line (IOSEVAN), ovarian carcinoma cell line with HER-2 gene

amplification (SKOV-3), nontumorigenic breast epithelial cell line (HBL100), and breast carcinoma cell lines with HER-2 gene amplification (BT474 and SKBR-3), were resolved by SDS-PAGE in 7.5% acrylamide gels and analyzed as a Western blot. The Western blot was probed with both antibodies specific for p68HER-2 (anti-ECDIIIa) and for p185HER-2 (anti-neu(C)).

Figure 5 shows that p68ECDIIIa binds to p185HER-2. In panel A: Two mg of SKBR-3 cells extracted in nondenaturing buffer were immunoprecipitated with 5 $\mu$ l anti-neu(N) specific for the N-terminal sequence of p68HER-2 and p185HER-2, or with 5  $\mu$ l anti-neu(C) specific for the C-terminus of p185HER-2 and then probed as a Western blot with both anti-ECDIIIa specific for p68HER-2 and with anti-neu(C) specific for p185HER-2. In panel B: 100  $\mu$ g of 17-3-1 cell extract were incubated in duplicate with 50  $\mu$ l packed volume of NiNTA agarose (Qiagen) coupled to 20  $\mu$ g of His-tagged ECDIIIa or to 20  $\mu$ g His-tagged CREB fragment in 200  $\mu$ l of wash buffer (20mM Tris pH 8.0, 300mM NaCl) at room temperature for 1 hr with shaking. The resin was then washed 4 times with 500  $\mu$ l of wash buffer and proteins were eluted by incubation with 50  $\mu$ l SDS-sample buffer at 100° C for 2 min. Eluted proteins were analyzed by Western blot analysis using antibodies against the C-terminus of p185HER-2, anti-neu(C). In panel C: Monolayers of  $\sim 10^5$  3T3 cells or HER-2 transfected 17-3-1 cells in 12 well plates were washed twice with PBS and then incubated with 0.5 ml of serum-free media with 1% BSA and 39, 75, 150, and 300 nM of purified recombinant His-tagged ECDIIIa for 2 hrs at 4 °C. Cells were washed 1 time in PBS containing 1% BSA and twice in PBS and then were extracted in denaturing buffer. Equal aliquots (20  $\mu$ g protein) were analyzed by western blotting with antibodies specific for ECDIIIa (anti-ECDIIIa) or, in the upper panel, with antibodies specific for p185HER-2 (anti-neu(C)).

Figure 6 shows that neither p68-rich conditioned media nor the ECDIIIa peptide stimulate tyrosine phosphorylation of p185HER-2. Monolayer cultures of  $\sim 10^5$  HER-2 transfected 17-3-1 cells were washed twice with PBS, incubated in serum-free media at 37 °C for 24 hrs, and then treated for 10 minutes with 75 or 150  $\mu$ M His-tagged ECDIIIa or with 50X CM from HEK-293 cells that secrete high levels of p68 or 50X CM from SKOV-3 cells that have no detectible p68HER-2. The treated cells were extracted with denaturing buffer containing the phosphotyrosine phosphatase inhibitor vanadate (2 mM) and 20  $\mu$ g/ml of cell extract protein from each sample were analyzed by Western blot analysis with monoclonal antibodies against phosphotyrosine (Sigma). The blot was stripped by incubation at 55° C for 30 min in 62.5 mM Tris pH 6.7, 2% SDS, and 100 mM 2 -mercaptoethanol and then reprobed with anti-neu(C) specific for p185HER-2.

Figure 7 shows that p68HER-2 inhibited anchorage independent growth of tumorigenic cells. SKOV-3 ovarian carcinoma cells and HER-2 transfected 17-3-1 cells were suspended in

media with 10% fetal bovine serum containing 0.3% agar (control conditions) to which was added 50X concentrated media conditioned by SKOV-3 cells (which contains no detectable p68HER-2 (-p68 CM)), or 50X concentrated media conditioned by HEK-293 cells (which contains 20 nM p68HER-2 (+p68 CM)). Five times 10<sup>3</sup> cells were plated in triplicate for each experimental condition onto a 0.5 ml layer of media containing 0.5% agarose in 12 well plates. The results shown are plotted as the mean and standard deviation of the number of colonies with more than 50 cells in triplicate wells counted at 21 days of incubation. Similar results were observed in three separate experiments.

Figure 8 shows the nucleotide and deduced amino acid sequence of HER-2 Intron 8.

Human genomic DNA was subjected to PCR using primers that flank intron 8. PCR parameters were 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 30 s, followed by 1 cycle of 72 °C for 7 min. A 410 bp product was gel purified and sequence in the forward an reverse directions. The sequence shown is the most common sequence found within intron 8 from about 15 different individuals. The positions of sequence variation that would result in amino acid substitutions are marked by Xs.

#### Detailed Description of the Invention

~~The present invention is based upon the initial discovery of an alternative HER-2 mRNA of 4.8 kb with a 274 bp insert identified as intron 8. The retained intron is in-frame and encodes 79 amino acids [SEQ ID NO. 1] followed by a stop codon at nucleotide 236. The alternative mRNA predicts a truncated HER-2 protein that lacks the transmembrane and intracellular domains and contains 419 amino acids [SEQ ID NO. 2]; 340 residues that are identical to the N-terminus of p185HER-2 and 79 unique residues at the C-terminus [SEQ ID NO. 1]. Using specific antibodies against either the novel 79 amino acid residue C-terminal sequence [SEQ ID NO. 1] or the N-terminus of p185HER-2, a 68 kDa protein product was identified [SEQ ID NO.2]. This 68 kDa protein is the product of an alternative HER-2 transcript, and is found in cell extracts and in extracellular media from several cell lines. Expression of the alternative transcript was highest in a nontransfected human embryonic kidney cell line.~~

The results presented here show expression of alternative HER-2 mRNA, which contains an additional 274 nucleotides, probably intron 8. Consistent with this finding, an alternative transcript of ~ 4.8 kb was detected in human fetal kidney tissue and in the human embryonic kidney cell line, HEK 293. Moreover, a transcript of 2.6 kb, which is the size expected if the sequence is retained in the 2.3 kb truncated HER-2 mRNA (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993), was detected in human fetal liver tissue by Northern blot analysis using a probe specific for the inserted sequence or for the HER-2 ECD coding sequence (Figure 2). The inserted sequence introduces a termination codon and predicts a novel 79 amino acid extension designated ECDIIIa at residue 340 of the



p185HER-2 protein. The predicted protein therefore lacks the transmembrane and intracellular domains, but contains subdomains I and II of the extracellular domain of p185HER-2. As predicted, a secreted protein that contains N-terminal sequence of p185HER-2 and the C-terminal extension provided by the inclusion of the novel sequence was detected (Figures 3 and 5). The ECDIIIa protein was found to be 68 kDa which is the approximate size expected of the protein encoded by the alternative transcript if the five N-linked glycosylation sites found in subdomains I and II of p185HER-2 are glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986).

The data presented herein demonstrate that p68HER-2 specifically binds to p185HER-2. The association with p185HER-2 may be conferred by the novel proline rich ECDIIIa domain rather than the N-terminal subdomains I and II of p68HER-2. While the HER-2 ECD, generated by *in vitro* deletion mutagenesis, also contains subdomains I and II, it does not associate with the extracellular domain of p185HER-2 unless engineered to enhance their proximity (Tzahar et al., *EMBO J.* 16:4938-4950, 1997; O'Rourke et al., *Proc. Natl. Acad. Sci. USA* 94:3250-3255, 1997; and Fitzpatrick et al., *FEBS Letters* 431:102-106, 1998). However, the unique ECDIIIa peptide binds with high affinity (nM concentrations) to p185HER-2 and to transfected 17-3-1 cells that overexpress p185HER-2 (Figure 5). Preferential binding of the ECDIIIa domain peptide to 17-3-1 cells indicates that secreted p68HER-2 interacts with the extracellular region of p185HER-2 at the cell surface. Therefore, p68HER-2 and fragments thereof appear to be a naturally occurring HER-2 binding protein, encoded by the HER-2 gene. In contrast to EGFR family ligands (Groenen et al., *Growth Factors* 11:235-257, 1994), p68HER-2 lacks an EGF homology domain and contains the first 340 amino acids of the receptor itself, p185HER.

Previously described putative HER-2 ligands were found to associate indirectly with p185HER-2 only in a heterodimer with an EGFR family member (Heldin and Ostman, *Cytokine Growth Factor Rev.* 7:33-40, 1996). Although it is possible that ECDIIIa binds indirectly to p185HER-2 through a coreceptor, this seems unlikely since detergent solubilized p185HER-2 was specifically and efficiently "pulled down" by immobilized ECDIIIa peptide (Figure 5B).

For all naturally occurring or engineered ligands for mammalian EGFR family members, binding is tightly coupled to stimulation of receptor dimerization and tyrosine phosphorylation (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; Dougall et al., *Oncogene* 9:2109-2123, 1994; and Groenen et al., *Growth Factors* 11:235-257, 1994). Although they bind, neither p68HER-2 nor the ECDIIIa peptide was found to activate p185HER-2. Activation was assessed in two different cell lines that differ in the extent of p185HER-2 tyrosine phosphorylation, transfected 17-3-1 cells as well as SKOV-3 ovarian carcinoma cells. Furthermore *in vitro* self-phosphorylation activity, which is enhanced in dimeric forms of p185HER-2 (Dougall et al., *Oncogene* 9:2109-2123, 1994; and Lin et al., *J. Cell. Biochem.* 49, 290-295, 1992), was not stimulated by p68HER-2 or ECDIIIa. Similarly, the Argos protein,

which is an extracellular inhibitor of the Drosophila EGF receptor and the only known antagonist of class I RTKs, did not simulate tyrosine phosphorylation of the receptor (Schweitzer et al., *Nature* 376:699-702, 1995). Likewise, Angiopoietin-2, a natural antagonist for the Tie 2 RTK, bound the endothelial receptor but failed to activate it (Maisonpierre et al., *Science* 277:55-60, 1997).

Without being bound by theory, since p68HER-2 occupies but does not activate, it could block dimerization of p185HER-2. By analogy, HER-2 ECD, when engineered to enhance its binding to RTKs, prevented the formation of productive dimers required for transphosphorylation and receptor activation thereby having a dominant negative effect (O'Rourke et al., *Proc. Natl. Acad. Sci. USA* 94:3250-3255, 1997). In contrast to the HER-2 ECD, soluble p68HER-2 exhibited strong binding to p185HER-2, yet also contains subdomain I and II of the ECD. Since subdomain I may be the low affinity, promiscuous ligand binding site required for recruitment of p185HER-2 into heteromeric complexes (Tzahar et al., *EMBO J.* 16:4938-4950, 1997), p68HER-2 could block this site and thereby obstruct recruitment of p185HER-2 into dimers. Alternatively, p68HER-2 could compete with an uncharacterized ligand for binding to p185HER-2. The tissue-specific expression of p68HER-2 in human fetal liver and kidney may function to modulate the extent to which p185HER-2 is occupied during development of these organs. Moreover, the overexpression of p185HER-2, relative to p68HER-2 in tumor cells with HER-2 gene amplification (Figure 3), could occur though a selective pressure based on overcoming the effects of a binding protein such as p68HER-2. Therefore, p68HER-2 is the first example of a naturally occurring p185HER-2 binding protein that may prevent activation of p185HER-2.

#### Pharmaceutical Composition

The present invention further provides a pharmaceutical composition for treating solid tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

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The inventive pharmaceutical composition, comprising either or both of the inventive polypeptides and/or monoclonal antibody, can be administered to a patient either by itself (complex or combination) or in pharmaceutical compositions where it is mixed with suitable carriers and excipients. Inventive polypeptide can be administered parenterally, such as by intravenous injection or infusion, intraperitoneal injection, subcutaneous injection, or intramuscular injection. Inventive polypeptide can be administered orally or rectally through appropriate formulation with carriers and excipients to form tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like. Inventive polypeptide can be administered topically, such as by skin patch, to achieve consistent systemic levels of active agent. Inventive polypeptide is formulated into topical creams, skin or mucosal patch, liquids or gels suitable to topical application to skin or mucosal membrane surfaces. Inventive polypeptide can be administered by inhaler to the respiratory tract for local or systemic treatment of cancers characterized by overexpressing HER-2.

The dosage of inventive polypeptide suitable for use with the present invention can be determined by those skilled in the art from this disclosure. Inventive polypeptide will contain an effective dosage (depending upon the route of administration and pharmacokinetics of the active agent) of inventive polypeptide and suitable pharmaceutical carriers and excipients, which are suitable for the particular route of administration of the formulation (*i.e.*, oral, parenteral, topical or by inhalation). The active inventive polypeptide is mixed into the pharmaceutical formulation by means of mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, entrapping or lyophilizing processes. The pharmaceutical formulations for parenteral administration include aqueous solutions of the inventive polypeptide in water-soluble form. Additionally, suspensions of the inventive polypeptide may be prepared as oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. The suspension may optionally contain stabilizers or agents to increase the solubility of the complex or combination to allow for more concentrated solutions.

Pharmaceutical formulations for oral administration can be obtained by combining the active compound with solid excipients, such as sugars (*e.g.*, lactose, sucrose, mannitol or sorbitol), cellulose preparations (*e.g.*, starch, methyl cellulose, hydroxypropylmethyl cellulose, and sodium carboxymethyl cellulose), gelatin, gums, or polyvinylpyrrolidone. In addition, a desintegrating agent may be added, and a stabilizer may be added.

#### Processes for Synthesizing p68 and 79 aa C Terminal Region

Polypeptide synthesis is done by a group of standard procedures for polypeptide synthesis

by sequential amino acids building through peptide synthesis equipment, following manufacturer's instructions for synthesizing peptides. Preferably, shorter polypeptides, of less than 100 amino acids, are best suited for the method of synthesis through sequential amino acid building of polypeptides. In addition, heterologous polypeptides can be expressed by transformed cells using standard recombinant DNA techniques to transform either prokaryotic or eukaryotic cells, provide appropriate growth media for their expression, and then purify the inventive polypeptide either from the media or from intracellular contents depending upon the type of cell used and its expression characteristics.

#### Methods for Treating Cancer with p68, 79 aa C Terminal Region, and Combinations

The present invention provides a method for treating a solid tumor characterized by overexpression of HER-2, or HER-2 variants (*see* Example 8) comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone. Preferably, the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer, prostate cancer, gastric carcinoma, cervical cancer, esophageal carcinoma, and colon cancer. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The p68HER-2 polypeptide described herein was found to bind to HER-2 and prevent signal transduction through the kinase domain. Without being bound by theory, the unique ECDIIIa domain mediates specific binding to p185HER-2 and the resulting interaction with p68ECDIIIa prevents p185HER-2 dimerization and subsequent signal transduction. Therefore, p68HER-2 functions as a HER-2 antagonist to prevent signal transduction by preventing dimerization as a necessary prerequisite for signal transduction. Thus, the mechanism of p68HER-2 as a HER-2 antagonist is different from the mechanism of binding agents, such as the 79 amino acid polypeptide described herein or a monoclonal antibody that binds to the EDC of HER-2. The inventive method provides that p68HER-2 inhibits tumor cell growth in tumors that overexpress HER-2 by providing a selective pressure for such tumor cells. Similarly, the HER-2

antagonists that are binding agents also inhibit tumor cell growth in tumors that overexpress HER-2 by providing selective pressure to such cells to prevent ligand binding to the ECD of HER-2 and prevent signal transduction even before potential dimerization.

#### Use of 79 aa C Terminal Region as a Targeting Molecule

5 The present invention further provides a method for targeting a therapeutic agent to solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD of HER-2). It was discovered that the 79 amino acid polypeptide [SEQ ID NO. 1] exhibited surprising high affinity binding properties to the ECD of HER-2. Moreover, the site of such binding is different and unaffected by the site of binding of a marketed humanized monoclonal antibody (Herceptin®). Therefore, the high binding affinity enables the 79 amino acid polypeptide to function as a targeting molecule to tumor cells expressing HER-2.

#### Anti-p68 Antibody as a Diagnostic/Prognostic Agent

15 The p68HER-2 ECDIIIa variant 3 (see TABLE 1, below) glycosylated polypeptide was expressed and used as an antigen for antibody production. Specifically, antibody specific for p68HER-2 was prepared by injecting rabbits with purified polyhistidine-tagged ECDIIIa variant 3 peptide, which is the same as the intron encoded novel C-terminus of p68HER-2, the domain that binds with high affinity to p185HER-2. The isolated polyclonal antibody detected pM quantities of ECDIIIa peptide or of p68HER-2 with high specificity (see Figures 3 and 5). Thus, an antibody specific for p68HER-2 is useful as a diagnostic agent for detecting p68HER-2 in bodily fluids and tumor tissues using diagnostic techniques, such as ELISA, immunoprecipitations, immunohistochemistry or Western analysis.

25 Antibodies that specifically recognize one or more epitopes of ECDIIIa, or epitopes of p68HER-2, or peptide fragments, and thus distinguish among ECDIIIa variants (see TABLE 1, below) are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single-chain antibodies, Fab fragments, F(ab').sub.2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The antibodies of the invention may be used, for example, in the detection of a particular p68HER-2 ECDIIIa variant in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients or tissue samples may be tested for the

presence of particular variants, or for abnormal amounts particular variants.

Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of particular p69HER-2 variants. Additionally, such antibodies can be used in conjunction with the cancer treatment methods described herein.

For the production of antibodies, various host animals may be immunized by injection with *e.g.*, polyhistidine-tagged ECDIIIa variant polypeptides, truncated ECDIIIa variant polypeptides, functional equivalents of the ECDIIIa variants or mutants of the ECDIIIa region. Such host animals may include but are not limited to rabbits, mice, hamsters and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (*Nature* 256:495-497, 1975; and U.S. Pat. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., *Immunology Today* 4:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030, 1983), and the EBV-hybridoma technique (Cole et al., *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Hybridomas producing mAb may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

Additionally, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855, 1984; Neuberger et al., *Nature*, 312:604-608, 1984; Takeda et al., *Nature*, 314: 452-454, 1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region (humanized).

Alternatively, techniques described for the production of single-chain antibodies (U.S. Pat. No. 4,946,778; Bird, *Science* 242:423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988; and Ward et al., *Nature* 334:544-546, 1989) can be adapted to produce

single-chain antibodies against ECDIIIa variant gene products. Single-chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab').sub.2 fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., *Science*, 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to particular ECDIIIa variants can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" the ECDIIIa variant, using techniques well known to those skilled in the art. (Greenspan & Bona, *FASEB J* 7 (5):437-444, 1993; and Nissinoff, *J. Immunol.* 147:2429-2438, 1991). For example antibodies which bind to an ECDIIIa variant and competitively inhibit the binding of p68HER-2 to HER-2 receptor can be used to generate anti-idiotypes that "mimic" the ECDIIIa variant and, therefore, bind and neutralize HER-2 receptor. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in cancer therapeutic regimens.

Alternatively, antibodies to particular ECDIIIa variants that can act as agonists or antagonists of the ECDIIIa variant activity can be generated. Such antibodies will bind to the ECDIIIa variant and modulate the activity of p68HER-2 vis-à-vis p185HER-2 receptor-mediated signal transduction. Such antibodies may be particularly useful for treating particular cancers and/or modulating tumor differentiation. Accordingly, the present invention further provides a method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2. The higher the ratio of p68HER-2:p185HER-2, the better the treatment prognosis.

#### ECDIIIa region Variants as Diagnostic/Prognostic Agents

Example 11 (below) shows that the human sequence of intron 8 is both proline-rich and polymorphic. Sequencing of genomic DNA from fifteen different individuals resulted in the identification of 10 variable sequence regions within Her-2 Intron 8. See SEQ ID NO:10; Figure

8, and Table 1. Figure 8 shows the most common nucleotide and corresponding polypeptide sequences of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an "X" in Figure 8) that result in nonconservative amino acid substitutions (see legend to TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue 341 in the "herstatin" sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999), is a polymorphic form that differs at amino acid residues #6 and #73 from the most commonly detected sequence shown here in Figure 8.

This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. An individual may, *inter alia*, be genetically heterozygous for two variants, homozygous for a given variant, or homozygous for a double variant. Both tumor progression and optimal treatment may vary depending upon the particular variants represented in a given individual.

This variability has both prognostic and diagnostic utility. The present invention shows that ECDIIIa-containing polypeptides can bind tightly to, and thus antagonize the HER-2 receptor. Such a specific, high-affinity interaction is dependent upon particular primary, secondary and tertiary structure of the ECDIIIa-containing polypeptide. The ECDIIIa region is proline-rich, and it is well known in the art that nonconservative substitution of proline residues, or other residues within a proline-rich sequence, in a given protein can have profound effects on its secondary and tertiary structure. Thus, the polymorphisms of the present invention are likely to embody significant structural, biochemical and biological differences relative to the most common polypeptide structure (shown in Figure 8). Structural differences among ECDIIIa variant proteins may include for example, differences in size, electronegativity, or antigenicity. Differences in biological properties among ECDIIIa variants might be seen *e.g.*, in the relative degree of cellular secretion, the nature and/or extent of modulation of the HER-2 receptor, pharmacokinetics (*e.g.*, serum half-life, elimination profile), resistance to proteolysis, N-linked glycosylation patterns, etc. These biological differences, in turn, would be expected to alter tumor progression and thus optimal treatment protocols. Thus, the knowledge that an individual contains a particular ECDIIIa variant or variants (*e.g.*, in individuals heterozygous for a given variant, or individuals with compound variants like variant 11 of Table 1), may, in itself, be prognostic of particular cancer susceptibility.



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The apparent genetic heterogeneity of ECDIIIa region means that the nature of the particular ECDIIIa variation carried by an individual may have to be ascertained using sequence identity assays prior to attempting genetic diagnosis of the patient. The analysis can be carried out on any genomic DNA derived from bodily fluids of the patient, typically a blood sample from an adult or child, but alternatively may be serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, and chorionic villi samples. It is expected that standard genetic diagnostic methods, such as hybridization or amplification assays, can be used. Either DNA or RNA, may, for example, be used in hybridization or amplification assays of biological samples to detect particular ECDIIIa variant sequences. Such sequence identity assays may include, but are not limited to, Southern or Northern analyses, single-stranded conformational polymorphism analysis, *in situ* hybridization assays, and polymerase chain reaction ("PCR") analyses. Such analyses may reveal both quantitative and qualitative aspects of ECDIIIa variant sequence expression. Such aspects may include, for example, point mutations, and/or activation or inactivation of gene expression. Standard *in situ* hybridization techniques may be used to provide information regarding which cells within a given tissue express a particular ECDIIIa variant sequence.

Preferably, diagnostic methods for the detection of ECDIIIa variant nucleic acid molecules involve contacting and incubating nucleic acids, derived from cell types or tissues being analyzed, with one or more labeled nucleic acid reagents, or probes, specific for particular ECDIIIa variants. More preferably, PCR, or reverse transcription PCR, can be utilized to identify nucleotide variation within the ECDIIIa domain. PCR reaction conditions should be chosen which optimize amplified product yield and specificity, and, additionally, produce amplified products of lengths that may be resolved utilizing standard gel electrophoresis techniques. Such reaction conditions are well known to those of skill in the art, and important reaction parameters include, for example, length and nucleotide sequence of oligonucleotide primers, and annealing and elongation step temperatures and reaction times. Following the PCR reaction, the PCR products can be analyzed by methods such as heteroduplex detection, cleavage of RNA-DNA hybrids using Rnase A, single-stranded conformational polymorphisms, and denaturing gradient gel electrophoresis.

Additionally, if the particular ECDIIIa sequence variant is known to add or remove a restriction site, or to have significantly altered the size of a particular restriction fragment, a protocol based upon restriction fragment length polymorphism ("RFLP") analysis may be appropriate.

ECDIIIa variants can also be analyzed at the expression level using sequence identity assays with bodily fluids derived from the patient, typically a blood sample from an adult or child, but may include serum, urine, lymph, saliva, tumor tissue, placental or umbilical cord

cells, amniotic fluid, and chorionic villi samples. Well-known sequence identity assays for analyzing expression include, but are not limited to, mRNA-based methods, such as Northern blots and *in situ* hybridization (using a nucleic acid probe derived from the relevant cDNA), and quantitative PCR (as described by St-Jacques et al., *Endocrinology* 134:2645-2657, 1994).

5 Polypeptide-based methods (e.g., including but not limited to western blot analysis) including the use of antibodies specific for the ECDIIIa variant of interest, as discussed above, could also be used. These techniques permit quantitation of the amount of expression of a given ECDIIIa variant, at least relative to positive and negative controls. Preferably, a battery of monoclonal antibodies, specific for different ECDIIIa epitopes or variants, could be used for  
10 rapidly screening cells or tissue samples to detect those expressing particular ECDIIIa variants, or for quantifying the level of ECDIIIa variant polypeptides. Preferred diagnostic methods for the quantitative or qualitative detection of ECDIIIa variant peptide molecules may involve, for example, immunoassays wherein particular ECDIIIa-containing peptides are detected by their interaction with anti-ECDIIIa variant specific antibodies. This can be accomplished for example,  
15 by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric, or fluorometric detection. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of ECDIIIa-containing peptides. Through the use of such procedures, it is possible to determine not only the presence of  
20 particular ECDIIIa-containing polypeptides, but also their distribution in the examined tissue.

Immunoassays for ECDIIIa variant polypeptides preferably comprise incubating a biological sample, such as the above-named bodily fluids, which have been incubated in the presence of a detectably labeled antibody capable of identifying ECDIIIa-containing peptides, and detecting bound antibody by any of a number of techniques well known in the art. The  
25 biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support that is capable of immobilizing soluble proteins, cells, or cell particles. The support may then be washed with suitable buffers followed by treatment with the detectably labeled anti-ECDIIIa variant specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The  
30 amount of bound label on the solid support may then be detected by conventional means.

Alternatively, anti-ECDIIIa variant specific antibodies can be detectably labeled by linking the same to an enzyme for use in an enzyme immunoassay or Enzyme Linked Immunosorbent Assay ("ELISA"). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably, a chromogenic substrate, in such a manner as to produce a  
35 chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not

limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and  
5 acetylcholinesterase.

The detection can be accomplished by calorimetric methods that employ a chromogenic substrate for the enzyme. Detection may also be accomplished visually by comparison of the extent of enzymatic reaction with appropriate standards. Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the  
10 antibodies or antibody fragments, it is possible to detect ECDIIIa-containing peptides through the use of a radioimmunoassay (RIA). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be  
15 detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as <sup>152</sup>Eu, or others of the lanthanide series. These metals can be attached to the antibody using such  
20 metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction.  
25 Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a  
30 bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The binding activity of a given lot of anti-ECDIIIa-variant specific antibody may be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine  
35 experimentation.

Accordingly, the present invention, including the unexpected discovery of a plurality of

variable sequence positions within the proline-rich ECDIIIa region, along with antibodies specific for particular ECDIIIa variants, provides for valuable prognostic and diagnostic information and assays.

Accordingly, the present invention further provides a method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2. The higher the ratio of p68HER-2:p185HER-2, the better the treatment prognosis. Preferably, the method for determining the prognosis of tumor treatment further comprises determining which particular ECDIIIa variants are present and optimizing tumor treatment in view of particular biochemical and biological properties among ECDIIIa protein variants.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; (b) determining whether a particular ECDIIIa variant sequence is present in the bodily fluid sample with a sequence identity assay; and (c) correlating the presence of the ECDIIIa variant sequence to cancer treatment and diagnosis using an historical database.

Preferably, the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; (b) determining whether an amount of an p68HER-2 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample, and determining a ratio between the amount of p185HER-2 ECD and a particular p68HER-2 ECDIIIa variant.

The present invention further provides for antibodies specific for ECDIIIa variants of the sequence in SEQ ID NO:1 or SEQ ID NO:2, below.

#### P68HER-2 as a Therapeutic Agent

Without being bound by theory, but it appears that p68HER-2 or ECDIIIa peptide inhibits the growth of tumor cells that overexpress HER-2 by binding to p185HER-2 at the cells surface. This hypothesis was examined by testing anchorage independent growth of cells in the presence or absence of p68HER-2 using cells that depend on p185HER-2 overexpression for their malignant growth, yet have little or no detectable p68HER-2. Anchorage independent growth of cells in soft agar was used as a predictive model for tumor cytotoxicity. This is a common and predictive procedure to examine transforming activity and reflects the tumorigenic and oncogenic potential of cells (DiFore et al., *Science* 237:178-182, 1987; Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987; and Baasner et al., *Oncogene* 13:901-911, 1996).

The effects of p68HER-2 on anchorage independent growth in soft agar was determined using SKOV-3 carcinoma cells and HER-2 transfected 17-3-1 cells, which are both tumorigenic and overexpress p185HER-2. The cells were suspended in media supplemented with fetal calf serum in the presence or absence of p68HER-2 and incubated for 21 days in a humidified incubator. Anchorage independent growth was quantitated by counting the number of colonies that contained more than 50 cells. Figure 7 shows that in the presence of p68HER-2, anchorage independent growth of both SKOV-3 cells and 17-3-1 cells was inhibited several fold. Accordingly, these data show that p68HER-2 is not just cytostatic, but cytotoxic and possibly apoptotic.

#### **Example 1**

This example provides the results from an experiment to investigate HER-2 mRNA diversity within the extracellular domain (ECD) coding sequence using polymerase chain reaction (PCR). A cDNA library from SKOV-3 cells (American Type Culture Collection (Rockville, MD) maintained in DMEM, supplemented with 10% fetal bovine serum and 0.05% gentamycin), an ovarian carcinoma cell line in which the HER-2 gene is amplified eight times (Tyson et al., *Am. J. Obstet. Gynecol.* 165:640-646, 1991) was examined using a forward primer specific for exon 1 (Tal et al., *Mol. Cell. Biol.* 7, 2597-2601, 1987) identical to nucleotides 142-

161 and a reverse primer complementary to nucleotides 1265-1286 in exon 9 (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). Briefly, The SKOV-3 cDNA library was provided by Origene Technologies, Inc. (Rockville, MD), and was prepared from RNA extracted from SKOV-3 cells. RNA was extracted from SKOV-3 cells grown to 80% confluence on 15 cm plates with  
5 TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol, to obtain total RNA. RNA was resuspended in 10mM Tris-EDTA, pH 8.0, for reverse transcription and cDNA library construction, or in RNA hybridization buffer (80% formamide, 40mM PIPES, 4 mM NaCl, 1mM EDTA, pH 7.5) for ribonuclease protection assay (RPA). RNA concentrations were determined spectrophotometrically at OD<sub>260</sub>. Poly A<sup>+</sup> mRNA was  
10 selected from total RNA using a mRNA extraction kit (Oligotex, Qiagen).

A product of ~1420 bp, determined to be HER-2-specific by Southern blotting, was approximately 270 bp larger than the expected size of 1144 bp from the previously described cDNA sequence (Coussens et al., *Science* 230:1132-1139, 1985). Briefly, the Southern blotting procedure transferred nucleic acids from agarose gels under vacuum (Bio-Rad Model 785  
15 Vacuum Blotter) in 0.4 M NaOH to Gene Screen Plus Hybridization Transfer Membrane (NEN Research Products, Boston, MA). Nucleic acids were fixed to membranes by UV crosslinking in a UV-Stratalinker (Stratagene, Inc., La Jolla, CA), and the membranes were blocked in hybridization buffer (50% formamide, 5X SSC, 1% SDS, 10 mg/ml herring sperm DNA) at 42 °C for 2 h. The membranes were hybridized at 42 °C for 16 h in hybridization buffer with 10<sup>7</sup>  
20 cpm of a 220 bp Kpn-HincII fragment from ECDIIIa cDNA labelled with (α-<sup>32</sup>P)dCTP (NEN Life Sciences) using a Random Prime DNA Labelling Kit (Boehringer Mannheim).

Templates were amplified in a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Cetus, Emeryville, CA) using the Expand High Fidelity PCR System (Boehringer Mannheim) with 1X High Fidelity PCR buffer containing 2.5 mM MgCl<sub>2</sub>, 5 μM of each primer, and 200 μM  
25 dNTPs. All primers were obtained from GIBCO BRL (Life Technologies). Numbering of nucleotide and amino acid residues is according to the HER-2 cDNA sequence reported by Coussens et al. (Coussens et al., *Science* 230:1132-1139, 1985). The HER-2 extracellular domain was targeted for amplification from an SKOV-3 cDNA library (Origene Technologies, Inc.) using a forward primer (A) identical to nucleotides (nt) 142-161 of HER-2 cDNA (5'-  
30 TGAGCACCATGAGCTGGC-3' [SEQ ID NO 3]), which spans the initiation codon (underlined) and a reverse primer (B) (5'-TCCGGCAGAAATGCCAGGCTCC-3' [SEQ ID NO 4]), which is complementary to HER-2 exon sequence at nt 1265-1286. Cycling parameters were: 94 °C, 30 sec; 58 °C, 45 sec; 68 °C, 3 min, for 30 cycles. The region spanning the alternative sequence (denoted ECDIIIa) from genomic DNA, was amplified using a forward  
35 primer (C) (5'-AACACAGCGGTGTGAGAAGTGC-3' [SEQ ID NO 5]) identical to HER-2 exon-specific sequence at nt 1131-1152 and the reverse primer (B) [SEQ ID NO. 4] on DNA

prepared as described (Bond et al., *FEBS Letters* 367:61-66, 1995) with cycling parameters: 94 °C, 30 sec; 62 °C, 30 sec; 72 °C, 60 sec, for 25 cycles.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to investigate the structure of mRNA containing the ECDIIIa sequence. First strand cDNA was reverse transcribed (Bond et al., *FEBS Letters* 367:61-66, 1995) using 5 µg RNA primed with 0.5 µg oligo-dT. To amplify the ECDIIIa insert and adjacent 5' HER-2 exon sequence, a forward primer (A) described above and a reverse primer (D) (5'-ATACCGGGACAGGTCAACAGC-3' [SEQ ID NO 6]) which is complementary to the 3'ECDIIIa-specific sequence were used. Cycling parameters were: 94 °C, 30 sec; 60 °C, 40 sec; 68 °C, 2 min, for 30 cycles.

Amplification of the ECDIIIa insert and adjacent 3' HER-2 exon-specific sequence was with a forward primer (E) (5'-TCTGGGTACCCACTCACTGC-3' [SEQ ID NO 7]) which is identical to the 5'ECDIIIa-specific sequence and contains a *Kpn*I restriction site and a reverse primer (F) (5'-TTCACACTGGCAGTCCAGACC-3' [SEQ ID NO 8]) which is complementary to HER-2 exon sequence at nt 3898-3919 and spans the termination codon (underlined). Cycling parameters were: 94 °C, 30 sec; 60 °C, 40 sec; 68 °C, 5 min, for 30 cycles.

The PCR product was subcloned and the nucleotide sequence was determined.

The results showed that the normal HER-2 coding sequence was present beginning with the 5' primer sequence and continued uninterrupted through nucleotide 1171. At this position, a 274 nucleotide insertion was found, followed by the expected coding sequence, including the 3' primer sequence. Analysis of the predicted protein product showed that the 274 nucleotide insertion encodes an extension of the known HER-2 protein, beginning at residue 340 (Coussens et al., *Science* 230:1132-1139, 1985), and introduces an in-frame stop codon 79 amino acids later (Figure 1). Comparison of the inserted nucleotides and their predicted amino acid sequence with sequences in Genbank showed no homologies. Examination of the 5' and 3' junctions of the divergent sequence revealed consensus splice donor and acceptor sites (Sharp, and Burge, *Cell* 91:875-879, 1997) and include a pyrimidine tract and potential branchpoint adenine residues near the 3'end of the insert sequence (Figure 1). Thus, the inserted sequence is likely to be an intron.

Inspection of the predicted amino acid sequence of the novel 79 amino acids [SEQ ID NO. 1] encoded by the inserted sequence shows a consensus N-linked glycosylation site and a high proline content of 19% (Figure 1). The inserted sequence was designated ECDIIIa since it is located at the boundary between subdomains II and III in the extracellular domain of the p185HER-2 sequence (Lax et al., *Mol. Cell. Biol.* 8:1831-1834, 1988). The insert sequence is in-frame with the adjacent 5' HER-2 exon sequence for 236 nt where there is a termination codon.

### Example 2

This example provides the results from experiments characterizing ECDIIIa as contiguous with HER-2 exons in the genome. To investigate the HER-2 gene structure in the

region of the ECDIIIa sequence, a forward primer, identical to nucleotides 763-785, and a reverse primer, complementary to nucleotides 1265-1286 of the HER-2 cDNA, were used in the PCR on human genomic DNA. The amplification product was anticipated to span exon 5 (Tal et al., *Mol. Cell. Biol.* 7:2597-2601, 1987) to an exon which is immediately 3' of the ECDIIIa sequence. Intron number and sizes were estimated based on PCR product sizes, restriction digest analysis, and partial sequence analysis of amplification products.

Next, human genomic DNA was examined using HER-2 exon-specific primers that directly flank the insert to determine the sequences immediately flanking the ECDIIIa sequence. A ~430 bp product was amplified from normal human genomic DNA and from genomic DNA extracted from carcinoma cell lines SKOV-3, SKBR-3 and BT474, all of which have HER-2 gene amplification (Kraus et al., *EMBO J.* 6:605-610, 1987) and were found to express ECDIIIa in their cDNA. The identities of the PCR products as HER-2 were verified by Southern blot analysis using the procedure described in Example 1. Nucleotide sequence analysis showed that the PCR product from human genomic DNA contained the ECDIIIa insert, flanked immediately on both sides by known HER-2 coding sequence; no mutations or rearrangements were seen. These data show that the ECDIIIa sequence represents a wholly retained intron, likely intron 8 based on the size of products amplified following intron 4 and on the location of intron 8 in the homologous EGFR gene and HER-3 gene (Lee and Maible, *Oncogene* 16:3243-3252, 1998).

### Example 3

This example shows that ECDIIIa is the only retained intron within the coding sequence of HER-2 mRNA. To determine whether additional introns were retained in the mRNA containing the ECDIIIa insert sequence, the reverse transcriptase-polymerase chain reaction (RT-PCR) was employed. First, a forward primer identical to 5' HER-2 cDNA sequence at 142-161 which spans the initiation codon, and a reverse primer complementary to the 3' ECDIIIa sequence were employed with SKBR-3 and SKOV-3 cDNA. A product of 1.3 kb was amplified, which is the size expected if the product contained no introns other than intron 8. Amplification of the 3'HER-2 coding sequence was then performed using a forward primer identical to 5' ECDIIIa sequence and a reverse primer complementary to 3'HER-2 cDNA sequence at nucleotides 3898-3919, which spans the p185HER-2 termination codon. A product of 2.9 kb was amplified, which is the size expected from the HER-2 cDNA if no additional introns were retained.

Further characterizations of both the 5'(1.3 kb) and 3'(2.9 kb) amplification products by restriction digest analysis and nucleotide sequencing confirmed the absence of additional retained introns. To determine the size of the products amplified when intron sequences are included, genomic DNA was used as a template for the PCR reactions, which resulted in products of approximately 10 kb for the 5' coding sequence and 5 kb for the 3' coding sequence.



These results indicate that the alternative HER-2 transcript, resulting from retention of an intron of 274 bp, was expected to be about 4.8 kb in size, assuming that the 5'untranslated (5'UTR) and 3'untranslated (3'UTR) regions are identical in size to the previously described ~4.5 kb HER-2 cDNA (Coussens et al., *Science* 230:1132-1139, 1985).

#### Example 4

This example illustrates the expression of a protein containing an ECDIIIa sequence. To assess whether the alternative sequence is translated into a protein product, the ECDIIIa sequence was expressed as a polyhistidine-tagged peptide in bacteria, purified the peptide by nickel-affinity chromatography, and raised antisera against the purified peptide. Briefly, the bacterial expression vector was prepared by amplifying the ECDIIIa sequence from the SKOV-3 cDNA library using primer E and a reverse primer complementary to the 3' end of the ECDIIIa insert sequence. The reverse primer contained a *Bam*H1 restriction site sequence, and was identical to that used for template construction in the RPA (described in examples 1 and 2). The PCR amplification product of ~280 bp was digested with *Kpn*I and *Bam*H1, gel purified (Qiaex II, Qiagen, Chatsworth, CA), and cloned into the pET30a vector, which encodes a six histidine tag at the amino-terminus of the expressed protein (Novagen, Madison, WI). The resulting expression vector, pET-ECDIIIa, was used for transformation of bacterial strain BL21.

To express the ECDIIIa protein product, BL21 cells transformed with the pET-ECDIIIa expression vector were grown in LB broth with 30 µg/ml Kanamycin for 4 h at 37 °C.

Expression was induced with 0.1 mM IPTG for 3 h and the harvested cells were lysed by sonication, and then centrifuged at 39,000 x g for 20 min. The supernatant was absorbed onto Ni-NTA agarose (Qiagen), by shaking for 60 min at room temperature. The resin was washed with ten volumes of wash buffer (10 mM Tris pH 7.9 and 300 mM NaCl), followed by ten volumes of wash buffer with 50 mM imidazole. The his-tagged ECDIIIa protein was eluted in wash buffer with 250 mM imidazole. The his-tagged protein, which was estimated to be approximately 90% pure by Coomassie Blue staining of gels, was used to generate and characterize antibodies.

Briefly, anti-ECDIIIa antisera were produced by Cocalico Biologicals, Inc. (Reamstown, PA) by injection of two rabbits with purified polyhistidine-tagged ECDIIIa peptide (described below). Polyclonal anti-*neu* (N) was produced against a peptide identical to amino acid residues 151-165 of p185HER-2 (Lin and Clinton, *Oncogene* 6:639-643, 1991). Polyclonal anti-*neu* (C) was made against a peptide identical to the last 15 residues of the carboxy-terminus of p185HER-2 (Lin et al., *Mol. Cell. Endocrin.* 69:111-119, 1990). Antisera from two immunized rabbits were characterized and found to contain antibodies of high titer that reacted with the purified ECDIIIa peptide.

A Western blot analysis examined whether SKBR-3 cells, which expressed the alternative

sequence in its cDNA, produced a protein that reacts with anti-ECDIIIa antibody. A 68 kDa protein from the cell extract and from the extracellular media reacted with anti-ECDIIIa antibody from two different rabbits diluted at least 20,000 fold, but not with preimmune sera. Inspection of the cDNA sequence of the alternative transcript (Figure 1) predicted a secreted protein product of 65-70 kDa if all 5 consensus N-linked glycosylation sites in the N-terminal p185HER-2 sequence were glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986).

If the 68 kDa ECDIIIa protein [SEQ ID NO. 2] is the translation product of the alternative HER-2 mRNA, then its N-terminal residues should be identical to the N-terminal 340 residues of p185HER-2. Therefore, cell extract from SKBR-3 cells was immunoprecipitated with anti-peptide antibody against an N-terminal sequence of HER-2, anti-neu (N) (Lin and Clinton, *Oncogene* 6:639-643, 1991) or with anti-ECDIIIa, and the immune complexes were examined by Western blot analysis with both antibodies. Briefly, three to 5  $\mu$ l of antisera were added to 2 mg of protein from cell lysates prepared in M-RIPA buffer (1% Nonidet P-40, 50 mM Tris pH 7.4, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 1% aprotinin), which had been centrifuged to remove nuclei. Immunoprecipitation was for 2 h with shaking at 4 °C as described (Lin et al., *Mol. Cell. Endocrin.* 69:111-119, 1990). The immune complexes were bound to Protein G Sepharose (Pharmacia) by incubation for 1 h at 4 °C with shaking, collected by centrifugation, and washed four times with M-RIPA. The proteins were released from the immune complex by incubation at 95° C for 2 min in SDS-PAGE sample buffer and resolved by SDS-PAGE in 7.5% gels (Mini-Protean II electrophoresis cell, Bio-Rad).

Western blotting was conducted following SDS-PAGE. Proteins were electroblotted onto nitrocellulose (Trans-blot, BioRad) using a semi-dry transfer unit (Bio-Rad) at 15 V for 20 min per gel (0.75 mm thick) equilibrated with 25 mM Tris pH 8.3, 192 mM glycine, 50 mM NaCl, and 20% methanol. The membranes were blocked with 5% nonfat dry milk at 25 °C for one hour. The blots were then incubated with primary antibody, washed twice for 15 min, and four times for 5 min with TBS-Tween (Tris-buffered saline containing 0.05% Tween), and then incubated for 40 min with goat anti-rabbit secondary antibody, conjugated to horseradish peroxidase (Bio-Rad), diluted 1:10, 000 in TBS-Tween. After incubation with secondary antibody, the membranes were washed as described above and reacted with chemiluminescent reagent (Pierce) and then were exposed to Kodak X-OMAT BLU film.

As expected, p68HER-2 was detected when anti-ECDIIIa was used for immunoprecipitation and for Western blot analysis. When anti-ECDIIIa was used for immunoprecipitation and anti-neu (N) was the probe in the Western blot, a 68kDa protein was detected, indicating that p68ECDIIIa contained the N-terminal sequence of p185HER-2. Further, anti-neu (N) precipitated p68HER-2, which was detected by probing with anti-ECDIIIa antibody. These results demonstrate that p68HER-2 contains both ECDIIIa and the N-terminal sequence

of HER-2.

Several other cell lines were examined for expression of p68ECDIIIa. The carcinoma cell lines which contained ECDIIIa sequence in their cDNA (BT474, SKOV-3) also had p68HER-2. Of several cell lines examined, HEK293 cells, derived from normal human embryonic kidney cells, expressed the highest levels of p68ECDIIIa in the cell extract and in the extracellular media, at about 5 to 10-fold higher amounts than SKBR-3 cells. In comparison to the carcinoma cell lines examined (SKBR-3, SKOV-3, and BT474) which overexpress p185HER-2, the HEK293 cells contained about 20 fold lower amounts of p185HER-2. Therefore, the relative proportion of p68HER-2 to p185HER-2 was at least 100 fold greater in HEK293 cells than in the three carcinoma cell lines studied. Reactivity with p68HER-2 as well as with a protein of ~120 kDa, particularly apparent in the HEK293 extracts, was blocked by preincubation of the antisera with purified ECDIIIa peptide demonstrating sequence-specific reactivity. The larger protein may be a dimer of p68HER-2. Therefore, p68HER-2 was expressed and secreted from several carcinoma cell lines and is at 5-10 fold elevated levels in HEK293.

#### Example 5

This example illustrates expression of an alternative HER-2 transcript containing the ECDIIIa intron sequence. Results of the RT-PCR analysis indicated that the ECDIIIa sequence was inserted into an otherwise normal-sized HER-2 mRNA. These data suggest an alternative transcript of ~4.8 kb. To examine the size and expression of the ECDIIIa alternative transcript, Northern blot analysis was conducted using an ECDIIIa-specific probe. Briefly, a template for antisense RNA probe synthesis was constructed from SKOV-3 cDNA by PCR amplification of a 389 bp sequence spanning the entire ECDIIIa insert sequence and containing adjacent 5'HER-2 exon sequence. The PCR was done using the forward primer C [SEQ ID NO. 5] that is identical to HER-2 cDNA sequence at nt 1131-1152 and a reverse primer (5'-GCACGGATCCATAGCAGACTGAG GAGG-3' [SEQ ID NO. 9]) which contains a 3' *Bam*H1 restriction endonuclease site and is complementary to the sequence spanning the 3' splice site of the ECDIIIa sequence. The PCR product was then digested with *Bam*H1, liberating a 375 bp fragment, which was cloned into pBluescript SK (Stratagene). The plasmid was sequenced by the Vollum Institute Core Sequencing Facility (Portland, OR) with m13 forward and reverse primers. An antisense RNA probe complimentary to the entire ECDIIIa sequence and to 87 nt of HER-2 exon sequence 5' to the insert was transcribed from 1 µg of linearized template using (α-<sup>32</sup>P) CTP, T7 RNA polymerase, and the T7/SP6 Riboprobe Synthesis System (Promega, Madison, WI). This probe was expected to protect a 370 nt fragment when hybridized with mRNA containing ECDIIIa and adjacent HER-2 exon sequence, and to protect an 87 nt fragment when hybridized with fully spliced HER-2 mRNA.

To prepare the RNA hybrids, 30 µg of RNA were hybridized with approximately 50,000 cpm of antisense RNA probe at 48 °C for 16 h. RNA hybrids were digested for 30 min at 37 °C with 40 µg/ml RNaseA (Boehringer Mannheim) and 2 µg/ml RNase T1 (Life Technologies) in a solution of 250 mM NaCl, 5 mM EDTA, and 10 mM Tris pH 7.5. Proteinase K (100 µg) (Life Technologies) in 20 µl 10% SDS was added to stop the digestion. Samples were extracted with acid phenol (pH 4.5; Life Technologies) and chloroform, precipitated with two volumes of 100% ethanol, and suspended in 5 µl of RPA sample buffer (88% formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue). Samples were denatured at 95° C for 10 min and electrophoresed on a 5% polyacrylamide/urea gel in TBE (89 mM Tris, 89 mM borate, 2 mM EDTA pH 8.3). Gels were dried under vacuum and subjected to phosphorimager analysis for quantitation of the protected fragments (IP Lab Gel, Molecular Dynamics).

An alternative transcript of approximately 4.8 kb was detected in HEK293 cells which expressed the highest levels of p68ECDIIIa. However an alternative transcript could not be detected by Northern analysis of the SKBR-3, BT474, or SKOV-3 carcinoma cell lines.

Therefore, the more sensitive ribonuclease protection assay (RPA) was employed to examine the expression levels of the alternative transcript relative to the fully spliced 4.5 kb transcript. RNA from ovarian (SKOV-3) and breast (SKBR-3 and BT474) carcinoma cell lines, which contained detectable levels of p68ECDIIIa, and a control cell line, 17-3-1, stably transfected with HER-2 cDNA, were hybridized with an antisense <sup>32</sup>P-labeled RNA probe which spanned the entire ECDIIIa (intron 8) sequence and 5' HER-2 exon sequence flanking intron 8. Following RNase digestion, electrophoresis, and autoradiography, a band of 370 nucleotides was detected in each cell line except for 17-3-1, which corresponds to the expected size protected by an ECDIIIa-containing HER-2 mRNA. In addition, an 87 nucleotide protected fragment was detected in all cells and is the size expected for the fully-spliced HER-2 message which is overexpressed by more than 100 fold in these carcinoma cell lines compared to normal control cell lines (Kraus et al., *EMBO J.* 6:605-610, 1987). The amounts of each protected fragment were quantitated and normalized for size to estimate the relative abundance of the alternative transcript, expressed as a percentage of the p185HER-2 mRNA. The alternative HER-2 mRNA with the ECDIIIa insert was at 4.2% the level of the fully spliced transcript in SKOV-3; 5.4% in SKBR-3, and 0.8% in BT474 cells.

### Example 6

This example shows that alternative transcripts containing the ECDIIIa insert were expressed in human embryonic kidney and liver. A Northern blot was conducted to examine whether an alternative transcript, which contains the ECDIIIa sequence, was expressed in normal human tissue. PolyA<sup>+</sup> mRNA from a variety of human fetal tissues prepared as a Northern blot was hybridized with a radiolabeled probe specific for the unique ECDIIIa sequence. A 4.8 kb

mRNA was detected in kidney and a 2.6 kb transcript was detected in liver (Figure 2). The 4.8 kb transcript likely corresponded to the full length 4.5 kb transcript with the 274bp insert and the 2.6 kb transcript may have corresponded to a previously described 2.3 kb alternative transcript (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993) with the 274bp ECDIIIa insert. When the blot was stripped and hybridized with a probe specific for the 5' HER-2 coding sequence, a broad band representing the 4.8 and 4.5 kb mRNAs was detected in fetal kidney tissues and the truncated 2.6 kb transcript was detected in liver showing that these alternative transcripts contain sequences that encode the HER-2 ECD. Because the inserted ECDIIIa sequence contained a termination codon, the same protein product may be produced from each of these mRNAs.

Several cell lines were also investigated for the ECDIIIa-containing alternative transcript by Northern blot analysis. The 4.8 kb alternative transcript was detected in the human embryonic kidney cell line, HEK-293 (Figure 2). Although the ECDIIIa sequence was detected by RT-PCR analysis of SKBR-3, BT474, and SKOV-3 carcinoma cell lines, which all contain HER-2 gene amplification, an ECDIIIa-containing alternative transcript could not be detected by Northern analysis of these cells. Therefore, the more sensitive ribonuclease protection assay (RPA) was employed using an antisense probe which spanned the entire ECDIIIa sequence and 5' HER-2 exon sequence flanking the ECDIIIa sequence. The alternative HER-2 mRNA with the ECDIIIa insert was detected at less than 5% of the fully spliced transcript in SKOV-3, SKBR-3, and BT474 cells. These findings show that two alternative transcripts containing the ECDIIIa sequence were expressed in a tissue-specific manner in normal human tissues, that the 4.8 kb alternative transcript was expressed in the HEK-293 cell line, and that the carcinoma cells with gene amplification express reduced amounts of the alternative transcript at less than 5% of the 4.5 kb HER-2 transcript.

### Example 7

This example illustrates expression of a protein containing the ECDIIIa sequence. To assess whether the alternative sequence was translated into a protein product, the ECDIIIa sequence, as a polyhistidine-tagged peptide in bacteria, was expressed and purified by nickel-affinity chromatography, and raised antisera against the purified peptide. The HEK-293 cells, which expressed the 4.8 kb ECDIIIa alternative transcript, were examined for expression of an ECDIIIa-containing protein by Western analysis. A 68 kDa protein from the cell extract and from the extracellular media reacted with the anti-ECDIIIa antibody (Figure 3) but not with preimmune sera and reactivity was blocked by preincubation of the antisera with purified ECDIIIa peptide (Figure 3). The larger protein of ~125 kDa detected in some cell extracts may be an aggregate of p68HER-2. The cDNA sequence of the alternative transcript (Figure 1) predicts a secreted protein product of 65-70 kDa if all 5 consensus N-linked glycosylation sites in

the N-terminal p185HER-2 sequence are glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986). Several other cell lines were examined for expression of p68ECDIIIa. The carcinoma cell lines which contained ECDIIIa sequence in their cDNA (BT474, SKOV-3, SKBR-3) also had detectable levels of p68HER-2.

#### Example 8

This example illustrates the expression of p68HER-2 relative to p185HER-2 was markedly reduced in carcinoma cell lines in which the HER-2 gene is amplified. Because the p68HER-2 mRNA was expressed at very low levels relative to the p185HER-2 mRNA in carcinoma cell lines with HER-2 gene amplification, the relative proportions of p68HER-2 and p185HER-2 proteins in several cell lines were examined with and without HER-2 gene amplification. Western blots were prepared and probed with both antisera specific for p68HER-2 and for p185HER-2. Figure 4 shows that p185HER-2 was readily detected in the carcinoma cells lines that have their HER-2 gene amplified about 8 times (Kraus et al., *EMBO J.* 6:605-610, 1987). However, there was not a corresponding elevation in p68HER-2. In comparison, p68HER-2 was the only HER-2 protein detected in the HEK-293, IOSEVAN, and HBL100 nontumorigenic cells, although p185HER-2 was expressed at very low levels in these cells (Kraus et al., *EMBO J.* 6:605-610, 1987) and was detected in overexposed blots. These data show that p68HER-2 was low in proportion to p185HER-2 in carcinoma cells with HER-2 gene amplification and suggests that a mechanism may exist to maintain low levels of p68HER-2 when p185HER-2 is overexpressed.

#### Example 9

This example illustrates that p68HER-2 and the ECDIIIa peptide specifically bind to p185HER-2. Because p68HER-2 is secreted and contains subdomains I and II identical to p185HER-2, in addition to a novel sequence, the possibility that p68HER-2 may interact with p185HER-2 was investigated. Antipeptide antibody against the N-terminus of p185HER-2 and p68HER-2, anti-neu (N), or antibody specific for p185HER-2, anti-neu(C), were used for immunoprecipitations of SKBR-3 carcinoma cells, which express low levels of p68HER-2 and overexpress p185HER-2. The immunoprecipitated material was prepared as a Western blot and probed with both anti-ECDIIIa specific for p68HER-2 and with anti-neu(C). Anti-neu (N) immunoprecipitated both p68HER-2 and p185HER-2 (Figure 5A). In addition, antibodies specific for the C-terminus of p185HER-2 immunoprecipitated p185HER-2 and coprecipitated p68HER-2 (Figure 5A), suggesting an interaction between the two proteins.

Since binding interactions between ECD sequences are very weak (Tzahar et al., *EMBO J.* 16:4938-4950, 1997; Fitzpatrick et al., *FEBS Letters* 431:102-106, 1998), the possibility that binding may be conferred by the novel proline rich ECDIIIa domain was examined. The unique 79 amino acid domain, purified as a His-tagged protein, was immobilized on nickel agarose and

used in a pull-down assay. For controls, two purified His- tagged peptides unrelated to ECDIIIa, a 600 residue fragment of the Wilson's disease membrane protein, and a 70 residue fragment containing the DNA binding domain of the CREB protein, were likewise immobilized on nickel agarose resin. The immobilized peptides were incubated with protein extracts prepared from HER-2 transfected 3T3 cells (17-3-1). Following extensive washes, the bound proteins were eluted and prepared as a Western blot which was probed with an antibody specific for p185HER-2. Equal amounts of His-tagged ECDIIIa peptide and control peptide were bound to the resin as confirmed by elution with 1M imidazole and Coomassie staining of the eluted material in SDS-gels. While no p185HER-2 was retained by resin without peptide or with control peptide, p185HER-2 was selectively retained by the ECDIIIa peptide (Figure 5B).

Since the ECDIIIa domain bound to p185HER-2 in a pulldown assay, the question of whether the ECDIIIa domain preferentially binds to cells that overexpress p185HER-2 was examined. This was investigated using monolayer cultures of 17-3-1 cells transfected with HER-2 compared to the parental 3T3 cells. The cells were incubated with different concentrations of the His-ECDIIIa peptide, washed, and extracted in denaturing buffer with protease inhibitors. To detect any bound peptide, the cell extracts were examined by Western blot analysis using antibodies specific for ECDIIIa. In addition, equal aliquots of the ECDIIIa peptide treated cells were reacted as a Western blot with antibodies specific for p185HER-2, demonstrating the overexpression of p185HER-2 in the transfected 17-3-1 cells. The ECDIIIa peptide preferentially bound to intact 17-3-1 cells at nM concentrations (Figure 5C) whereas little or no peptide was found to bind to equivalent amounts of parental 3T3 cells suggesting a specific interaction with the extracellular domain of p185HER-2.

#### Example 10

Effect of p68ECDIIIa and the ECDIIIa peptide on tyrosine phosphorylation of p185HER-2 was examined. Tyrosine phosphorylation of RTKs is the initial indication of ligand activation and signal transduction. Tyrosine phosphorylation in 17-3-1 cells treated with different amounts of the purified ECDIIIa peptide, with conditioned media (CM) from HEK293 cells that contained high levels of p68HER-2 (Figure 2A), or with control, conditioned media from SKOV-3 cells that had no detectable p68HER-2 were examined. There was no increase in the tyrosine phosphorylation signal at 10 minutes (Figure 6) or 2 hrs of treatment with His-ECDIIIa or with concentrated CM suggesting that p185HER-2 was not activated. Neither p68HER-2-containing CM nor the ECDIIIa peptide detectably altered the phosphotyrosine signal corresponding to p185HER-2 from SKOV-3 cells in which p185HER-2 tyrosine phosphorylation levels were low. Additionally, p68HER-2 and the ECDIIIa peptide had no discernable effect on *in vitro* self-phosphorylation activity of p185HER-2 immunoprecipitated from 17-3-1 cell extracts. These results support the conclusion that p68HER-2 did not activate p185HER-2 signal transduction.

### Example 11

This example illustrates that the sequence of intron 8 is polymorphic. Intron 8 of the human HER-2 gene is alternatively retained in mRNA, and encodes a novel 79-residue domain at the C-terminus of a part of the extracellular domain of p185HER-2. The product, “herstatin,” of the alternative transcript with the retained intron functions as an autoinhibitor of the HER-2 oncogene. The intron 8 encoded domain, alone, was shown to bind with nM affinity to p185HER-2. (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999).

Additionally, polymorphisms in the nucleotide and deduced amino acid sequence of intron 8 in the HER-2 gene were identified by sequencing genomic DNA from 15 different individuals. Figure 8 and SEQ ID NO:1 show the most common nucleotide and corresponding amino acid sequences, respectively, of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an “X” in Figure 8) that result in nonconservative amino acid substitutions (*see* legend to TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2.. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue #341 in the “herstatin” sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) is a polymorphic form that differs at amino acid residues #6 and #73 from the most commonly detected sequence shown here in Figure 8. This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. Some identified variants are summarized in Table 1.



TABLE 1

	X(4)	X(14)	X(17)	X(47)	X(54)	X(62)	X(106)	X(161)	X(191)	X(217)
Variant 1	T									
Variant 2		C								
Variant 3			T							
Variant 4				A						
Variant 5					A					
Variant 6						C, T, A				
Variant 7							A			
Variant 8								G		
Variant 9									T	
Variant 10										C
Variant 11			T							C

**Table 1.** Sequence variants in the intron-8 encoded domain found in the human population

(based on 15 different individuals). Sequence variants 1-11 are listed, showing the base changes at a particular “X” positions relative to that found in the most common DNA sequence shown in Figure 8. The numbers in parenthesis after each X correspond to the nucleotide position in the DNA sequence shown in Figure 8. The DNA sequence variants listed here correspond to the variable amino acid positions (“Xaa”) of SEQ ID NO:1 as follows: X(4) to Xaa(2); X(14) to Xaa(5); X(17) to Xaa(6); X(47) to Xaa(16); X(54) to Xaa(18); X(62) to Xaa(21); X(106) to Xaa(36); X(161) to Xaa(54); X(191) to Xaa(64); X(217) to Xaa(73); and to the variable amino acid positions of SEQ ID NO:2 as follows: X(4) to Xaa(342); X(14) to Xaa(345); X(17) to Xaa(346); X(47) to Xaa(356); X(54) to Xaa(358); X(62) to Xaa(361); X(106) to Xaa(376); X(161) to Xaa(394); X(191) to Xaa(404); X(217) to Xaa(413). The specific amino acid changes (relative to the most common DNA sequence of Figure 8) for the variable amino acid positions in SEQ ID NO:1 are: Variant 1, Xaa(2)(Thr→Ser); Variant 2, Xaa(5) (Leu→Pro); Variant 3, Xaa(6) (Pro→Leu); Variant 4, Xaa(16) (Leu→Gln); Variant 5, Xaa(18) (Met→Leu); Variant 6, Xaa(21) (Gly→Asp, Alu or Val); Variant 7, Xaa(36) (Leu→Ile); Variant 8, Xaa(54) (Pro→Arg); Variant 9, Xaa(64) (Pro→Leu); Variant 10, Xaa(73) (Asp→Asn), and Variant 11, Xaa(6) (Pro→Leu) and Xaa(73) (Asp→Asn). The same substitutions apply to the corresponding variable amino acid positions in SEQ ID NO:2.